Nitrate is a negative signal for fructan synthesis and the fructosyltransferase-inducing trehalose inhibits nitrogen and carbon assimilation in excised barley leaves

R. Morcuende, S. Kostadinova*, P. Pérez, I. M. Martín del Molino, R. Martínez-Carrasco

Instituto de Recursos Naturales y Agrobiología de Salamanca, CSIC. Apartado 257, 37071 Salamanca, Spain

**Present address: Department of Agrochemistry and Soil Science, Agricultural University, 12 Mendeleev str., 4000 Plovdiv, Bulgaria*

Correspondence: Rafael Martínez-Carrasco: Tel: +34923272202; Fax: +34923219609; e-mail: rafaelmc@usal.es

Summary

- Fructan biosynthesis in barley has been shown to be up-regulated by sugar signalling and down-regulated by nitrogen. We have investigated the relationship between these two regulations.
- Excised third-leaves of barley were fed nitrate or glutamine under two light intensities. Other leaf blades were supplied in the dark for 24 h with nitrate and trehalose in the presence of validamycin A, a trehalase inhibitor.
- In the light, nitrate, but not glutamine, decreased fructan contents and sucrose:fructan 6-fructosyltransferase protein without affecting the levels of sucrose and other carbohydrates. In darkened leaves, trehalose increased and nitrate decreased the fructan contents and total sucrose:fructosyltransferase activity without altering the concentration of sucrose. The effect on fructan contents of trehalose disappeared, while that of nitrate still remained in subsequent incubations in water under light. Trehalose decreased and nitrate increased the light- and CO_2-saturated rate of photosynthesis without significantly affecting the initial Rubisco activity. Trehalose feeding decreased the activation of Nitrate Reductase and amino acid levels, and blocked the positive effect of nitrate on the maximal activity of this enzyme.
- The results indicate that nitrate, and not a down-stream metabolite, is a negative signal for fructan synthesis, independent from the positive sugar signalling and overriding it. Trehalose signalling inhibits nitrogen and carbon assimilation, at the same time inducing fructosyltransferase activity.

Keywords: barley, fructan, fructosyltransferase, nitrogen, nitrate, regulation, trehalose

Abbreviations: 1-SST: sucrose:sucrose 1- fructosyltransferase; 6-SFT: sucrose:fructan 6-fructosyltransferase; Rubisco: Ribulose -1, 5- bisphosphate carboxylase oxygenase; RuBP: Ribulose-1,5- bisphosphate.

Introduction

Fructans are fructose polymers present in many plants, including cereals and grasses, as reserve carbohydrates. Fructans are also claimed to enhance the tolerance of plants to cold and drought (Ritsema & Smeekens 2003). Fructan synthesis is dependent on sucrose accumulation and it has been demonstrated there is a threshold concentration of sucrose for fructan production (Pontis 1970; Pollock et al. 2003), accompanied by an induction of gene expression and enzyme activity, so that sucrose is not only a substrate, but also an effector for induction of fructosyltransferase activity (Wagner et al. 1986; Pollock & Cairns 1991). Among the fructosyltransferases, sucrose:fructan 6-fructosyltransferase (6-SFT) is particularly strongly induced by the external application of sucrose, the level of mRNA for this enzyme increasing conspicuously at the same time (Müller et al. 2000). The cloning of the barley 6-SFT and its induction by light and sucrose has recently been reported (Nagaraj et al. 2001). Trehalose, a disaccharide homologous to sucrose, induced the activity of sucrose:sucrose fructosyltransferase (SST) but did not stimulate fructan accumulation (Wagner et al. 1986). Trehalose had stimulatory effects on 6-SFT activity and, to a lesser extent, on 6-SFT mRNA, even in the presence of validoxylamine A, a potent trehalase inhibitor (Müller et al. 2000). Since hexoses phosphorylated by hexokinase but not or weakly metabolized did not increase fructan synthesis, it was concluded that the regulation of fructan synthesis in barley leaves is independent of hexokinase and is probably based on the sensing of sucrose (Müller et al. 2000). It has been shown that protein kinases and phosphatases are involved in the induction of fructosyltransferases (Martinez Nöel et al. 2001). Like sucrose, trehalose (+ validamycin A) stimulates the activity of sucrose synthase in the roots, while

glucose has no marked effect (Müller et al. 1998), and also induces the expression of the *ApL3* gene for ADP-glucose pyrophosphorylase and starch synthesis (Wingler et al. 2000), so that it can replace sucrose as a regulatory compound in sugar-mediated gene expression.

Stress conditions, such as drought (De Roover et al. 2000), low temperatures (Tognetti et al. 1990; Pérez et al. 2001), or nitrogen deficiency (Wang & Tillberg 1996), can rapidly enhance fructan accumulation, together with an induction of 1-SST or 6-SFT (Wang & Tillberg 1996; Van den Ende et al. 1999; Wang et al. 2000; De Roover et al. 2000). In barley leaves, the abundance of 6-SFT transcript was strongly, and that of 1-SST only slightly and transiently stimulated by nitrogen deficiency, a dramatic decrease in 6-SFT mRNA levels being observed during nitrogen re-supply. It was concluded that 6-SFT plays a key role in the regulation of fructan accumulation under nitrogen deficiency (Wang et al. 2000). It has been suggested that in source leaves of barley the increase in fructan accumulation under nitrogen deficiency may be due to the restricted export to sinks, and retention of sucrose in source tissues (Wang & Tillberg 1996). While drought-induced fructan synthesis in roots and leaves of *Cichorium intibus* (De Roover 2000), and low temperature-induced fructan synthesis in tall fescue leaves (Pérez et al. 2001) were associated with increases in glucose, fructose and sucrose, in barley leaves under nitrogen deficiency SST activity was not correlated with the relatively steady sucrose levels (Wang & Tillberg 1996). So far, studies on effects of nitrogen on fructan synthesis have been carried out using nitrate (Wang et al 2000) or ammonium nitrate (Van den Ende et al. 1999) as the source of nitrogen, and the question as to which nitrogen compound actually modulates fructan synthesis has not yet been addressed.

During nitrate assimilation, carbohydrate synthesis is decreased and more carbon is converted through glycolysis to phosphoenolpyruvate and enters organic acid metabolism (Stitt et al. 2002). Nitrate assimilation is closely integrated with carbon metabolism and nitrate is known to affect several enzymes of carbon and nitrogen metabolism (Stitt et al. 2002). Thus, nitrate induces the expression of a number of genes coding for enzymes of organic acid synthesis, while inhibiting the expression of the regulatory subunit of ADP-glucose pyrophosphorylase and decreasing starch synthesis; on the other hand, there is no evidence for an inhibition of sucrose synthesis caused by nitrate (Scheible et al. 1997a). Glutamine can decrease carbohydrate contents by diverting more carbon for the synthesis of α-ketoglutarate and glutamate (Morcuende et al. 1998), and this may limit fructan synthesis. Increased fructan synthesis under nitrogen deficiency would then be another element in the coordination of carbon and nitrogen metabolism. In turn, carbohydrates play a regulatory role in nitrogen metabolism. Sucrose and glucose increase the activation state of Nitrate Reductase (Morcuende et al. 1998), sugars or sugar-phosphates being the signals regulating protein kinase(s) and phosphatase involved in modulation of Nitrate Reductase activity (Kaiser & Huber 2001). Moreover, low sugar concentrations repress *NIA* expression, overriding signals derived from nitrate and nitrogen metabolism (Klein et al. 2000). Carbohydrate status also affects nitrogen metabolism at sites downstream of nitrate assimilation, with a general inhibition of amino acid synthesis when sugars are low (Matt et al. 1998; Morcuende et al. 1998). In addition, a depression of carbon assimilation has been observed during active fructan synthesis (Martínez-Carrasco et al. 1993; Pérez et al. 2001; Jenkins et al. 2002), accompanied by low levels of phosphorylated intermediates (Pérez et al. 2001).

Since fructosyltransferases are induced by high levels of sucrose, this work investigated whether enhanced fructan synthesis under nitrogen deficiency is mediated by carbohydrate status. Excised barley leaves were induced to synthesize fructan under continuous illumination and effects of nitrogen supply on this synthesis and on carbohydrate concentration were examined. In another approach, the effect of nitrogen on fructan synthesis was tested with the low carbohydrate levels of darkened leaves, in combination with trehalose (and validamycin A), which is known to induce the enzymes for fructan synthesis without being a substrate for this synthesis (Wagner et al. 1986). These leaves were subsequently transferred to light to assess effects of dark incubations on fructan synthesis when carbon supply from photosynthesis is resumed. We have compared nitrate and glutamine feeding to detached leaves, to know which nitrogen compound acts as a regulator in fructan synthesis. The contents of amino acids and the activities of two key enzymes in nitrogen and carbon assimilation, Nitrate Reductase and Rubisco, respectively, were analyzed to assess possible changes in these assimilations coordinated with an alteration of fructan synthesis.

Material and Methods

Plant material

Seeds of barley (*Hordeum vulgare* L. cv. Clarine) were sown in 2 l pots (25 seeds per pot) containing perlite which were placed in a growth room with 350 μmol m^{-2} s^{-1} photon flux density (fluorescent and incandescent), 22/16 ºC day/night temperature and 70 % relative humidity in a 16 h photoperiod. The plants were supplied with water and a nutrient solution containing 10 mM KNO_3 and micronutrients (Martinez-Carrasco et al. 1998). When third-leaves were fully expanded, they were cut with a sharp scalpel and immediately placed with the cut end in water. After 30 min, the leaves were transferred to 5 cm high petri dishes with the cut end dip in water or the test solutions through slots in the covers.

Treatment of excised leaves

The leaves were incubated in water, 10 mM glutamine, 1 mM KNO_3 or 10 mM KNO_3 under continuous 150 or 350 μmol m^{-2} s^{-1} light intensity for 7 or 24 h, with the growth room temperature and humidity conditions indicated above. There were 3 replicates, each consisting of 4 petri dishes (2 leaves per dish). For dark incubations, the leaves were fed for 24 h with all the factorial combinations of two nitrate levels (0 or 100 mM KNO_3) and three trehalose levels (0, 100 mM o 200 mM) supplemented with 10 μM validamycin A (Duchefa, Haarlem, The Netherlands) to inhibit trehalase activity and the release of glucose which can be metabolized (Müller et al. 2000). Temperature was kept at 22 ºC and humidification was stopped to favour stomatal aperture and transpiration, thus facilitating uptake of solutions in darkness (Wagner et al. 1986). There were 3 replicates, with 7 petri dishes and 2 leaves each.

Thereafter, the cut ends of leaves in 3 petri dishes per replicate were washed with water and the solutions were replaced by water. The leaves were then placed under light (350 µmol m^{-2} s^{-1}) for 5 hours. At the end of each incubation period, the leaves were cut above the dish cover and rapidly transferred *in situ* to liquid nitrogen and stored at –80 ºC until analysed. To assess the effects of incubation solutions on leaf hydration, after the dark incubations 2 leaves were cut in sections, weighed and floated on water for 24 h in the laboratory before recording the fully turgid weight. The leaves were then dried at 60 ºC for 24 h and the dry weights determined. The relative water content was estimated as (fresh weight - dry weight) x100/ (turgid weight - dry weight). Before harvesting the leaves kept in water under light after 24 h dark incubations, photosynthesis was measured in 3 replicate leaves with an infrared gas analyser (CIRAS-2, PP Systems, Hitchin, Herts., UK), with 1500 µmol mol^{-1} CO_2 and 1000 µmol m^{-2} s^{-1} irradiance.

Carbohydrate determination

Subsamples (about 100 mg FW) of leaves stored in liquid nitrogen were extracted three times in 1 ml 80 % ethanol -ethanol–*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes)-KOH (pH 7.5) at 60 ºC for 30 min, followed by three extractions in 1 ml water at 80 ºC for 30 min, centrifuging at 13000 g for 10 min and decanting the supernatant each time. The extracts were pooled and brought to 10 ml with 40 % ethanol. Subsamples (1 ml) were vacuum dried and resuspended in water. Glucose and fructose were analyzed with a spectrophotometric assay coupled to NADP reduction (Jones et al. 1977). Since commercial invertases hydrolyze fructans (Koroleva et al. 1998), sucrose was analyzed by incubating another aliquot with sucrase (Megazyme, Bray, Ireland) in sodium maleate buffer (pH 6.5) for 30 min,

then measuring glucose and fructose released as above, and substracting free glucose and fructose. Fructans were hydrolyzed with fructanase (Megazyme) – which according to the manufacturer completely hydrolyzes cereal fructans - in sodium acetate buffer (pH 4.5) for 30 min before analyzing glucose and fructose with the method described; to these, free- and sucrose-hexoses were substracted. Amino acids were analyzed according to Hare (1977). The insoluble residue of the ethanol and water extracts was homogenized with water and autoclaved (ap Rees et al. 1977) and starch was measured as glucose in the supernatant after incubation at 37 ºC with amyloglucosidase (essentially free of β-glucanase activity) and α-amylase (Roche Diagnostics, Barcelona, Spain). Tests were performed for specificity of sucrase and fructanase against sucrose and fructan (kestose, kestotetraose and kestopentaose - Megazyme) standards, and for complete hydrolysis to hexoses of these carbohydrates. Including small, representative amounts of metabolites in the extraction medium, recovery was above 87% of the amount added.

Fructosyl transferase assay

For analysis of fructosyltransferase activity with the high concentrations of extract and substrate required for fructan polymerization (Cairns et al. 1999), 0.2 g of frozen leaves were extracted in a chilled mortar with 1.2 ml of 50 mM citrate-phosphate buffer (pH 5.5), 2 mM dithiothreitol (DTT) and 2 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 17000 g at 5 ºC for 5 min, PEG-6000 to a final concentration of 40 % was added to the supernatant, which was allowed to stand on ice for 45 min before centrifugation at 17000 g at 5 ºC for 5 min. The supernatant was then discarded and the precipitate dissolved in 120 µl of extraction buffer. The dissolved extract was incubated with 600 mM sucrose (final concentration) in a total

volume of 170 µl at 30 ºC. At 5, 15, 30, 60 and 90 min the reaction was stopped in 20 µl aliquots by heating at 90 ºC for 5 min. After cooling and suspending in water, glucose and fructose were analyzed. Fructosyltransferase activity was estimated as the sucrose-dependent production of glucose minus fructose, and invertase activity as the sucrose-dependent production of fructose (although it also includes fructosyl transfer to water by fructosyltransferases) (Lüscher and Nelson 1995).

Nitrate reductase and Rubisco

Subsamples of frozen leaves were extracted and analyzed for Nitrate Reductase activity in the absence or presence of Mg^{2+} as described (Scheible et al. 1997b), with either 10 mM magnesium acetate (selective activity) or 5 mM EDTA (total activity) in the assay buffer; the activation state is given by the ratio of both activities. For Rubisco activity assays, a procedure based on that described by Lilley and Walker (1974), as modified by Ward and Keys (1989) and Sharkey *et al.* (1991), was followed. Aliquots of the frozen leaves were ground in a mortar with liquid nitrogen and extracted with 100 mM N,N-Bis(2-hydroxyethyl)glycine (Bicine)-NaOH (pH 7.8), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2% polyvinylpolypyrrolidone (PVPP) (w/v). An aliquot of the whole extract was used to determine chlorophyll contents (Arnon, 1949) and the remainder was centrifuged at 13000 *g*. The total time from extraction to the assay of initial Rubisco activity was less than 2.5 min. Activity was assayed by adding extract to a mixture of 100 mM Bicine (pH 8.2), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM KCl, 1 mM Ribulose-1,5- bisphosphate (RuBP), 0.2 mM NADH, 5 mM ATP, 5 mM creatine phosphate, 52 units/ml phosphocreatine kinase, 12 units/ml phosphoglycerate kinase, 11 units/ml glyceraldehyde 3-phosphate dehydrogenase and recording the decrease in absorbance at 340 nm minus 400 nm

for 40-60 s, at a stoichiometry of 2:1 between NADH oxidation and RuBP carboxylation. To assay total Rubisco activity, an aliquot of the extract was incubated with $NaHCO_3$ and $MgCl_2$ for 10 min at room temperature prior to the addition of coupling enzymes and NADH; the reaction was started by adding RuBP. The activation state was estimated as initial activity as a percentage of total activity. Commercial coupling enzymes suspended in ammonium sulphate were precipitated by centrifugation and dissolved in 20 % glycerol (Sharkey *et al.,* 1991). With the assay buffer described, the initial lag in the reaction reported by others (Ward & Keys, 1989; Sharkey *et al.,* 1991) was not observed. Checks were made for the linearity of enzyme activities over time and for the proportionality between rate and amount of extracts.

Protein electrophoresis and blotting

Proteins were extracted from frozen leaf subsamples ground to a fine powder in 50 mM citrate-phosphate buffer (pH 5.5), 1 mM EDTA, 5 mM 6-aminocaproic acid, 2 mM benzamidine, 5 mM β-mercaptoetanol, 1 mM phenylmethylsulphonyl fluoride (PMSF) for 20 min on ice, followed by centrifugation at 12000 g at 4 ºC for 25 min. Protein content was measured in the decanted supernatant (Bradford 1976) and five volumes of cold acetone were added to an aliquot containing 500 µg protein, which was left overnight in the freezer. The sample was then centrifuged at 12000 g at 4 ºC for 15 min and the acetone allowed to evaporate. The precipitate was dissolved in 65 mM Tris-HCl (pH 6.8), 25 % glycerol, 0.6 M β-mercaptoetanol, 2.5 % sodium dodecyl sulphate (SDS) and 0.01% bromophenol blue at 96 ºC for 7 min. The samples were cooled at room temperature and loaded on a 12.5 % sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Martín del Molino et al. 1995). The proteins were electro-blotted to PVDF membranes using a semi-dry transfer unit (trans blot, BioRad). Quantitative transfer of proteins was checked by staining of the transferred gels with Coomassie Brilliant Blue. The membranes were then incubated in 5 % low fat milk, 20 mM Tris (pH 7.5), 500 mM NaCl (TBS) for 6 h. After washing with 20 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween-20 (TTBS), the membranes were probed with antibodies raised against 6-SFT. Inmunodetection was performed with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad Laboratories, Madrid, Spain) which reacted with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitro blue tetrazolium (NBT) to yield the indigo-derived precipitate. The stained bands were quantified with a laser-scanning densitometer (Molecular Dynamics, Amersham Biosciences, Barcelona, Spain). Both the antibodies and the synthetic peptides HIPLRQGTHARHAE and VHEMDSAHNQLSNE, corresponding to the 430-443 and 598-611 amino acids of the 49 kD and 23 kD subunits, respectively, of 6-SFT (Sprenger et al. 1995) were made by the Service for Antibody Production, Instituto de Biología Molecular, CSIC (Barcelona, Spain).

Statistical methods

Analyses of variance were performed as in a fully randomized factorial experiment according to Snedecor and Cochran (1967), and from these the standard errors of differences were derived. These errors were preferred to the standard errors of means as estimates of the treatment effects.

Results

Incubation of excised leaves in the light

Carbohydrate and amino acid contents

The contents of carbohydrates and enzyme activities in leaves attached to the plant at the beginning of the incubations are included in the figures for comparison and will not be described further. Fructan concentrations increased with time of incubation and light intensity (Fig. 1), showing a positive response to carbon supply and probably also to induction of activity of fructan synthesis enzymes (Wagner et al. 1986; Pollock & Cairns 1991). In contrast, fructan decreased with 10 mM nitrate as compared to water, and relatively more so with a higher light intensity and thus with higher carbon supply; 1 mM nitrate had little effect on fructan contents. Glutamine (10 mM) had no significant effect on fructan contents. The responses of glucose and fructose to incubation conditions were similar to those described for fructan, except for a negative effect of glutamine as compared to water on the concentration of these sugars after 24 h. It may be noted that glucose contents were 2-7 fold higher that those of fructose. Sucrose did not show the same response to nitrogen as fructan and hexoses. Although sucrose increased in response to light intensity, as the other carbohydrates did, it did not decrease. After 7 h incubations, an increase in light intensity had a negative, rather than positive effect on starch contents, possibly because the flux of carbon was directed towards the synthesis of other compounds for the first hours. Starch increased with light intensity after 24 h, and was not significantly affected by nitrate or glutamine. The duration of incubations strongly increased amino acid contents in leaves in 10 mM nitrate and, specially, in glutamine (Fig. 2). Rising the light intensity increased the amino acid

contents at 24 h in leaves fed with 10 mM nitrate and glutamine, but not in those fed with 1 mM nitrate or water. Feeding 10 mM nitrate and, to a great extent glutamine, increased the amino acid contents.

Enzyme activities and amounts

Maximal Nitrate Reductase activity did not change significantly with time of incubation or light intensity (Fig. 3). Nitrate increased the activity, more at 10 mM than at 1 mM concentration, and increased the activation of the enzyme similarly with the two concentrations. Glutamine did not affect the activity or the activation of this enzyme.

Rubisco activity increased with light intensity, specially at 7 h, and in leaves incubated under high light, it decreased from 7 to 24 h (Fig. 4). Rubisco activation state also increased with light intensity and, generally, with time of incubation. Nitrate and glutamine did not affect the activity or the activation state of Rubisco.

Antibodies were raised against the peptides corresponding to amino acids 430-443 (49 kDa subunit) and 598-611 (23 kDa subunit) of 6-SFT, which are protein regions with little homology with invertases (Sprenger et al. 1995). The target of the antibody against 430-443 residues was close to Rubisco large subunit in the Western blots, and thus the antibody against the 23 kD subunit, which had a high titer, was preferred. In leaves incubated for 7 h, the densitometric analysis of the proteins separated by SDS-PAGE and further Western blot (Fig. 5) indicated that the amount of 6-SFT was increased by light intensity and decreased by nitrate, more so at 10 mM than at 1 mM concentration.

Incubation of excised leaves in the dark

The regulation of fructan synthesis by nitrogen was further investigated in incubations of leaves in the dark, in the absence of carbon supply from

photosynthesis. The effect of nitrate was compared with that of trehalose, which induces the expression of genes for fructan synthesis; validamycin A was added to prevent trehalose hydrolysis to glucose which can be metabolized (Müller et al. 2000). The possible osmotic effects of the solutions were assessed by measuring the relative water content of leaves. Compared to leaves incubated in water or 100 mM nitrate alone (99.3 % ± 0.13 and 98.7 % ± 0.16, respectively), leaves incubated in trehalose had lower relative water contents (92.1 % ± 3.0 and 89 % ± 3.0 for 100 and 200 mM trehalose, respectively), but within the range for well-watered plants, ruling out that leaves experienced water deficits.

Carbohydrate and amino acid contents

The concentration of fructan, as those of glucose, fructose and starch at the end of the 24 h dark incubation increased with trehalose and decreased with nitrate as compared with controls in water (Fig. 6). Sucrose, in contrast, was not significantly affected. When carbon in the various carbohydrates in leaves at the end of 24 h dark incubations was summed and substracted from the initial value under light, it was observed that 61% of the large increase in fructan content when 200 mM trehalose plus validamycin A was fed was not accounted for by mobilization of these carbohydrates in the dark. Analysis of the commercial products showed no sucrose or hexose impurities which could be used for fructan synthesis, and no trehalose hydrolysis by sucrase or fructanase (data not shown). Therefore, either trehalose was metabolized, which is unlikely (Wagner et al. 1986; Müller et al. 2000), or alternative, not analysed compounds were used as a carbon source for the synthesis of fructan. After 5 h incubations in water under light of leaves previously incubated in the various solutions in the dark, the stimulating effect on fructan content of 200

mM trehalose feeding had disappeared, suggesting that fructan had been mobilized, while nitrate still decreased fructan contents when it had been fed alone or in combination with 200 mM trehalose; feeding 100 mM trehalose in the dark had little effect on fructan contents after the subsequent incubation under light. As at the end of the dark period, under light glucose and fructose increased with trehalose and decreased with nitrate preincubations relative to water, while starch was higher with trehalose incubations but, in contrast with glucose and fructose, increased with nitrate. After 5 h in water in the light, sucrose decreased in leaves previously incubated in trehalose and nitrate relative to water. Amino acids increased in the incubation period in water under light relative to the dark levels (Fig. 7). The amino acid contents decreased with trehalose as compared to water, both after 24 h dark and the subsequent 5 h in water under light. Nitrate had no effect on amino acid contents after 24 h in darkness, while it increased these contents after 5 h in water under illumination.

Enzyme activities

After 24 h incubations in the dark, the sucrose-dependent fructosyltransferase activity (Fig. 8) increased with trehalose concentration; nitrate decreased this activity both when supplied alone or in combination with 200 mM trehalose, although the effect was not observed with 100 mM trehalose. Invertase activity showed similar responses to the treatments, but they did not reach statistical significance (data not shown).

In contrast with fructosyltransferase activity, the activation state of Nitrate Reductase significantly decreased with trehalose feeding as compared to water in the incubations in water and light after darkness (Fig. 9). Nitrate increased the maximal Nitrate Reductase activity only in the absence of trehalose.

Rubisco activity and activation state were not significantly affected by trehalose feeding in the dark and at the end of the subsequent incubation period in water and light (Fig. 10). Nitrate increased total Rubisco activity at the end of 24 h incubations in darkness. Following 5 h in water under light, the positive effect of nitrate on total Rubisco activity disappeared in leaves previously incubated in 200 mM trehalose.

The effects of dark pre-incubations on photosynthesis were examined in leaves in water 5 h into the light period (Fig. 11). Photosynthesis was inhibited as the trehalose concentration increased, and tended to increase with nitrate alone or combined with 100 mM trehalose, but this increase was suppressed by 200 mM trehalose.

Discussion

The increases in fructan concentration in response to time of exposure to, and intensity of light in this study are consistent with previously observed responses to an artificial prevention of export and consequent accumulation of soluble carbohydrates in illuminated excised leaves (Housley & Pollock 1985; Wagner et al. 1986; Müller et al. 2000). Under these conditions of high carbohydrate contents, inductive of fructan synthesis (Pollock et al. 2003), nitrate feeding decreased fructan accumulation. The question as to which nitrogen compound modulates fructan synthesis has not been addressed to date. By comparing the additions of nitrate and glutamine we show that fructan synthesis is inhibited by nitrate, and not by down-stream metabolites in nitrogen assimilation. Glutamine decreased sucrose contents, possibly by diverting more carbon for synthesis of α-ketoglutarate and glutamate (Morcuende et al. 1998), but did not decrease fructan levels. This rules out a beneficial effect of glutamine as compared to nitrate through an increase in substrate availability for fructan synthesis.

In contrast with glutamine, nitrate did not decrease sucrose concentrations, in association with the increase in amino acids, nor increased the allocation of photosynthetic carbon to starch synthesis. High glucose:fructose ratios in detached leaves incubated under light have also been observed in previous studies following induction of fructan synthesis (Wagner et al. 1986; Koroleva et al. 1998; Wang et al. 2000). This could result from fructan synthetic activity, which would incorporate fructose to the fructan pool, releasing free glucose from sucrose (Koroleva et al. 1998). This suggests that nitrate does not increase fructan degradation, which would release free fructose and decrease the glucose:fructose ratio but, instead inhibits

fructan synthesis. Moreover, nitrate also decreased fructan contents with the low carbohydrate status of darkened leaves. This ruled out that the inhibitory role of nitrate was mediated by decreased levels of sugars, which are a substrate and signal for fructan synthesis (Wagner et al. 1986; Pollock & Cairns 1991; Sprenger et al. 1995; Müller et al. 2000; Lu et al. 2002). Therefore, in intact plants nitrate would not decrease fructan simply by increasing carbohydrate export to sinks with an enhanced growth (Wang & Tillberg 1996). Our Western blot analyses show that, under light, nitrate inhibited fructan synthesis through a decrease in the amount of the enzyme 6-SFT, which is consistent with the decreased mRNA for 6-SFT after re-supplying nitrogen found by Wang et al. (2000). Inhibition of fructosyltransferase activity by nitrate in the dark confirms that the effect was due to a reduction of at least one enzyme of fructan synthesis. Since nitrate inhibited the positive effect on fructosyltransferase activity of trehalose, which increases the expression of 6-SFT mRNA (Müller et al. 2000), it possibly repressed gene expression for this enzyme. The trehalose-induced accumulation of fructan in the dark is at variance with the results of Müller et al. (2000) and may derive from undetermined carbon sources rather than from trehalose metabolization. These results indicate that nitrate was a negative signal for fructan synthesis, independent from carbohydrate level and overriding carbohydrate signalling. Nitrate has been shown to be a regulatory signal for several enzymes for nitrogen uptake and assimilation and organic acid and carbohydrate metabolism (Stitt et al. 2002). In particular, the other major storage carbohydrate, starch, has been shown to decrease with nitrate through decreased ADP-glucose pyrophosphorylase transcripts. The coordination of nitrogen and carbon metabolism includes, therefore, fructan synthesis, which is regulated by nitrate in an independent and antagonist way from carbohydrate signalling.

At variance with fructosyltransferase activity, the activity of Rubisco showed no significant changes in response to trehalose. This resembles the result by Lu et al. (2002) that *6-SFT* is induced by sugars while the messages for Rubisco small subunit (*RbcS*) and chlorophyll a/b binding protein (*Cab*) are not. The decrease in photosynthesis in leaves pre-incubated in 200 mM trehalose in the dark was not due, therefore, to limitations in carboxylation but to restricted photosynthetic electron transport or phosphate recycling to chloroplasts. Decreased carbon assimilation could account for the decrease in total carbon in the analyzed carbohydrates and in sucrose contents in leaves previously incubated in trehalose, as well as for the small stimulation of fructan synthesis under light in these leaves. Wingler et al. (2000) found a decrease in sucrose after trehalose feeding, and Müller et al. (2001) found decreased sucrose and starch levels in above-ground organs of Arabidopsis in response to the supply of validamycin, which inhibited trehalase activity and increased trehalose levels. However, based on the increase in non-structural carbohydrates in the shoot of Arabidopsis, Wingler et al. (2000) concluded that trehalose does not inhibit photosynthesis. The reasons for this discrepancy are not known. The enhanced fructan synthesis and depressed photosynthesis after trehalose feeding is reminiscent of our previously observed depression of photosynthesis during active fructan synthesis (Martínez-Carrasco et al. 1993; Pérez et al. 2001), associated with decreased levels of phosphorylated intermediates (Pérez et al. 2001). In a clonal line of transgenic white clover accumulating high levels of fructan, photosynthesis and carbohydrate contents were also decreased (Jenkins et al. 2002).

While inhibiting fructan synthesis and accumulation under light, nitrate increased Nitrate Reductase activity and amino acid levels, as observed in previous studies (Morcuende et al. 1998). Notably, the increase in fructan synthesis with dark

incubations in trehalose occurred along with a decrease in activation and thus activity of Nitrate Reductase and with decreased amino acid levels. This inhibitory effect of trehalose on enzyme activation is the opposite to the enhancement caused by feeding sucrose and glucose which can be metabolized to sucrose (Morcuende et al. 1998), a stimulation which can be due to the inhibition of Nitrate Reductase kinase by hexose monophosphates (Kaiser and Huber 2001). Deactivation of Nitrate Reductase by trehalose in darkened leaves and leaves illuminated after darkness was associated with increases in contents of glucose and hexose monophosphates (not shown) in both situations, and with sucrose levels unchanged in the dark and decreased in the light. In view of this result, a role for trehalose mediated by these sugars is difficult to envisage. Trehalose might act through a different, still unknown mechanism. In addition, feeding trehalose in the dark inhibited the stimulatory effect of nitrate on total Nitrate Reductase activity at the end of the dark period and after 5 h in the light, which could suggest that the induction of gene expression for this enzyme by nitrate (Srivastava 1980; Pouteau et al. 1989; Cheng et al. 1992) was repressed. Trehalose, therefore, acted in opposing ways to induce fructan synthesis and, at least transiently, starch synthesis in agreement with preceding studies (Wingler et al. 2000), and to repress carbon and nitrate assimilation. Similar opposing trends are observed in intact plants, in which nitrate inhibits the synthesis of fructan (Wang &Tillberg 1996) and starch (Scheible et al. 1997a) while increasing nitrogen assimilation.

In conclusion, we have shown that nitrate acts as a signal decreasing 6-SFT protein and, probably, mRNA, and that during trehalose-induced fructan synthesis nitrate assimilation and photosynthesis are depressed.

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Figure legends

Fig. 1 Concentration of carbohydrates in barley (*Hordeum vulgare*) leaves incubated in 150 μmol m^{-2} s^{-1} (D) or 350 μmol m^{-2} s^{-1} (B) light intensity in water (W), 10 mM glutamine (G) or 1 mM (1N) or 10 mM nitrate (10N) for 7 h (open columns) or 24 h (tinted columns). Black columns, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations. Values are means of three replicates. Vertical bars represent twice the SE of the difference between means for solutions (a) and for incubation times and light intensities (b).

Fig. 2 Amino acid concentrations in barley (*Hordeum vulgare*) leaves incubated in 150 μmol m^{-2} s^{-1} (D) or 350 μmol m^{-2} s^{-1} (B) light intensity in water (W), 10 mM glutamine (G) or 1 mM (1N) or 10 mM nitrate (10N) for 7 h (open columns) or 24 h (tinted columns); black column, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations.

Fig. 3 Activity and activation state of nitrate reductase in barley (*Hordeum vulgare*) leaves incubated in 150 μmol m^{-2} s^{-1} (D) or 350 μmol m^{-2} s^{-1} (B) light intensity in water (W), 10 mM glutamine (G) or 1 mM (1N) or 10 mM nitrate (10N) for 7 h (open columns) or 24 h (tinted columns); black columns, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations.

Fig. 4 Activity and activation state of Rubisco in barley (*Hordeum vulgare*) leaves incubated in 150 μmol m^{-2} s^{-1} (D) or 350 μmol m^{-2} s^{-1} (B) light intensity in water (W), 10 mM glutamine (G) or 1 mM (1N) or 10 mM nitrate (10N) for 7 h (open

columns) or 24 h (tinted columns); black columns, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations.

Fig. 5. Western blot (A) and densitometric analysis (B) of abundance of the enzyme sucrose:fructan 6-fructosyltransferase in barley (*Hordeum vulgare*) leaves incubated in 150 μmol m^{-2} s^{-1} (D) or 350 μmol m^{-2} s^{-1} (B) light intensity in water (W), 10 mM glutamine (G), 1mM (1N) or 10 mM nitrate (10N) for 7 h. Western blots is one of the three replicates. The molecular weights of the protein markers are indicated on the left; arrow indicates the sucrose:fructan 6-fructosyltransferase band.

Fig. 6. Concentration of carbohydrates in barley (*Hordeum vulgare*) leaves incubated in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (0N) or combined with 100 mM nitrate (100N) in the dark for 24 h (open columns) and subsequently incubated in water under 350 μmol m^{-2} s^{-1} light intensity for 5 h (tinted columns); black columns, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations. Vertical bars represent twice the SE of the difference between means for trehalose and nitrate at 24 h (a and b, respectively) and at 24+ 5 h (c and d, respectively).

Fig. 7. Amino acid concentrations in barley (*Hordeum vulgare*) leaves incubated in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (0N) or combined with 100 mM nitrate (100N) in the dark for 24 h (open columns) and subsequently incubated in water under 350 μmol m^{-2} s^{-1} light intensity for 5 h (tinted columns); black columns, intact leaves under 350 μmol m^{-2} s^{-1} irradiance at the start of incubations. Vertical bars represent twice the SE of the difference between means for

trehalose and nitrate at 24 h (a and b, respectively) and at 24+ 5 h (c and d, respectively).

Fig. 8. Fructosyltransferase activity with sucrose as substrate in barley (*Hordeum vulgare*) leaves incubated in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (open columns) or combined with 100 mM nitrate (tinted columns) in the dark for 24 h. Vertical bars represent twice the SE of the difference between means for trehalose (a) and nitrate (b).

Fig. 9. Activity and activation state of Nitrate Reductase in barley (*Hordeum vulgare*) leaves incubated in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (0N) or combined with 100 mM nitrate (100N) in the dark for 24 h (open columns) and subsequently incubated in water under 350 µmol m^{-2} s^{-1} light intensity for 5 h (tinted columns); black columns, intact leaves under 350 µmol m^{-2} s^{-1} irradiance at the start of incubations. Vertical bars represent twice the SE of the difference between means for trehalose and nitrate at 24 h (a and b, respectively) and at 24+ 5 h (c and d, respectively).

Fig 10. Activity and activation state of Rubisco in barley (*Hordeum vulgare*) leaves incubated in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (0N) or combined with 100 mM nitrate (100N) in the dark for 24 h (open columns) and subsequently incubated in water under 350 µmol m^{-2} s^{-1} light intensity for 5 h (tinted columns) ; black columns, intact leaves under 350 µmol m^{-2} s^{-1} irradiance at the start of incubations. Vertical bars represent twice the SE of the difference between means

for trehalose and nitrate at 24 h (a and b, respectively) and at 24+ 5 h (c and d, respectively).

Fig. 11. Photosynthetic carbon assimilation with 1500 µmol mol^{-1} CO_2 and 1000 µmol m^{-2} s^{-1} irradiance in barley (*Hordeum vulgare*) leaves incubated in water under 350 µmol m^{-2} s^{-1} light for 5 h following incubations in water (0), 100 mM (100T) or 200 mM trehalose (200T) alone (open columns) or combined with 100 mM nitrate (tinted columns) in the dark for 24 h. Vertical bars represent twice the SE of the difference between means for trehalose (a) and nitrate (b).

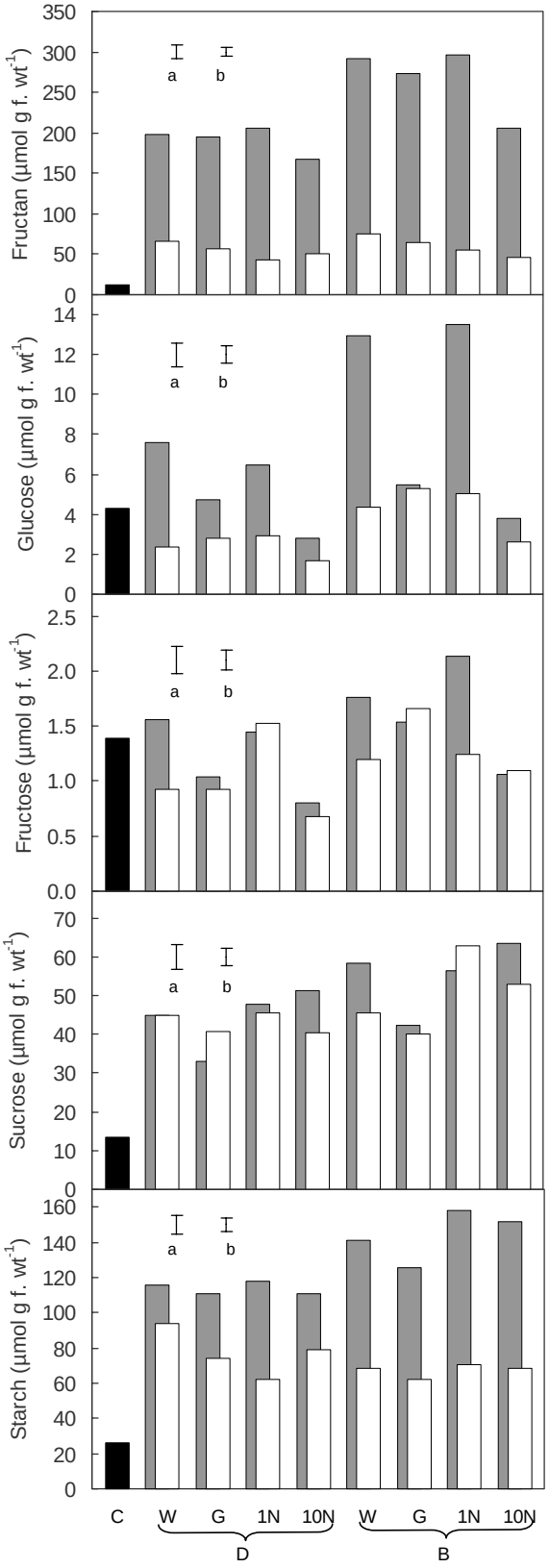
Fructan (µmol g f. wt-1)
Glucose (µmol g f. wt-1)
Fructose (µmol g f. wt-1)
Sucrose (µmol g f. wt-1)
Starch (µmol g f. wt-1)
a
b
C
W
G
1N
10N
D
B

Fig. 1

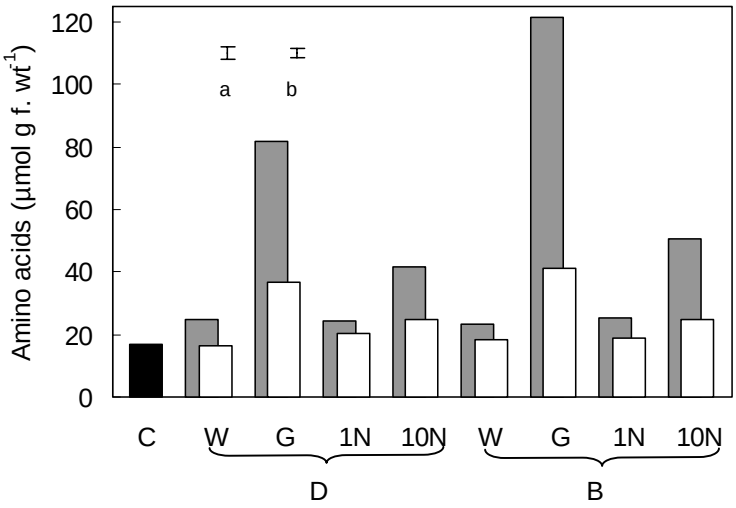
Amino acids (µmol g f. wf-1)
120
100
80
60
40
20
0
a
b
C
W
G
1N
10N
W
G
1N
10N
D
B

Fig. 2

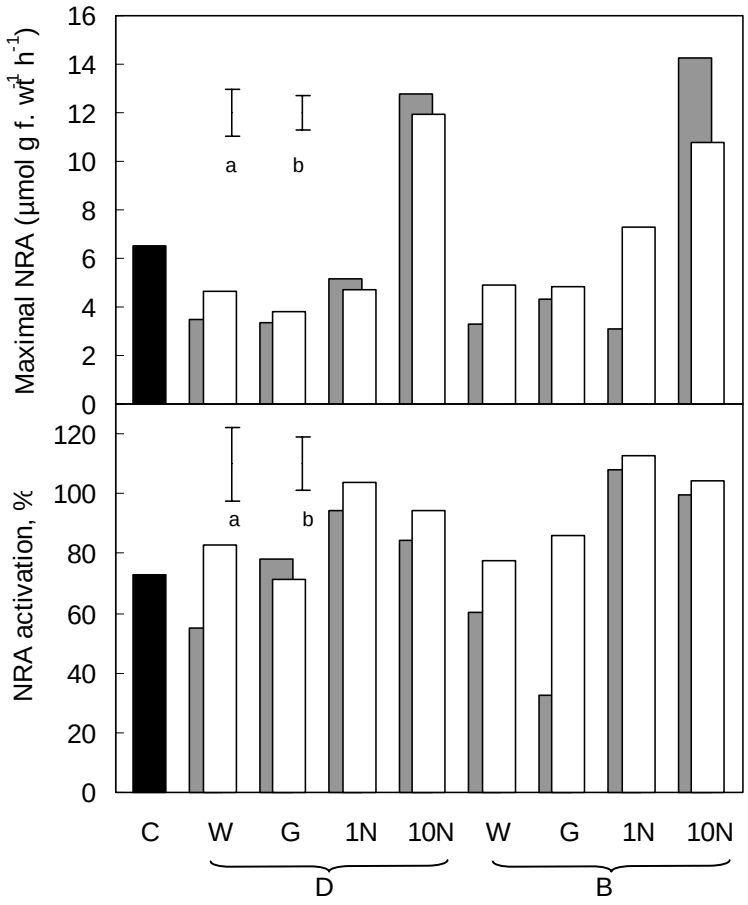
Maximal NRA (µmol g f. wt^{-1} h^{-1})
16
14
12
10
8
6
4
2
0
a
b
NRA activation, %
120
100
80
60
40
20
0
a
b
C
W
G
1N
10N
W
G
1N
10N
D
B

Fig. 3

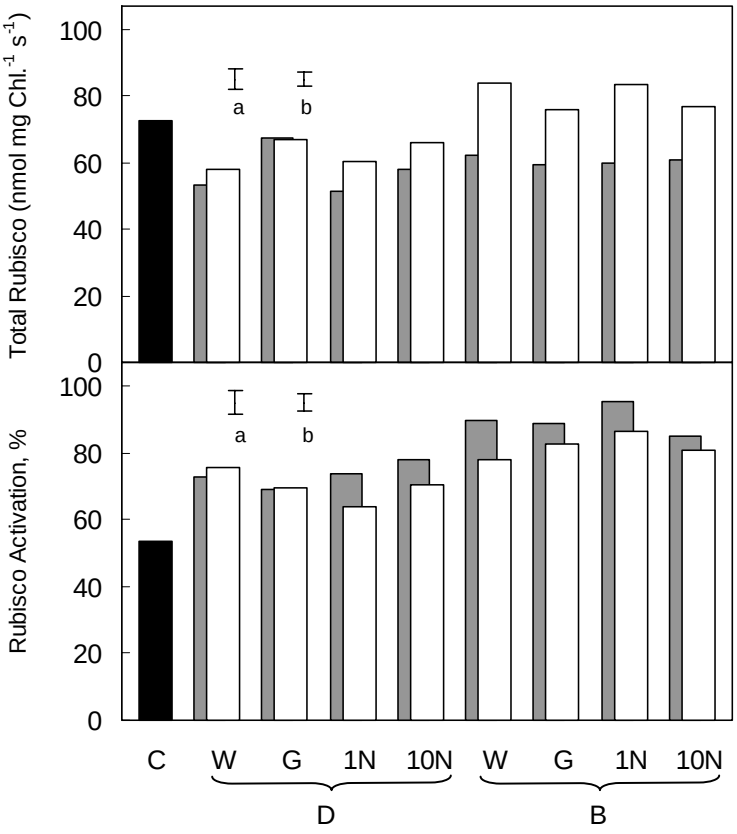
Total Rubisco (nmol mg Chl.-1 s-1)
Rubisco Activation, %
100
80
60
40
20
0
a
b
C
W
G
1N
10N
W
G
1N
10N
D
B

Fig. 4

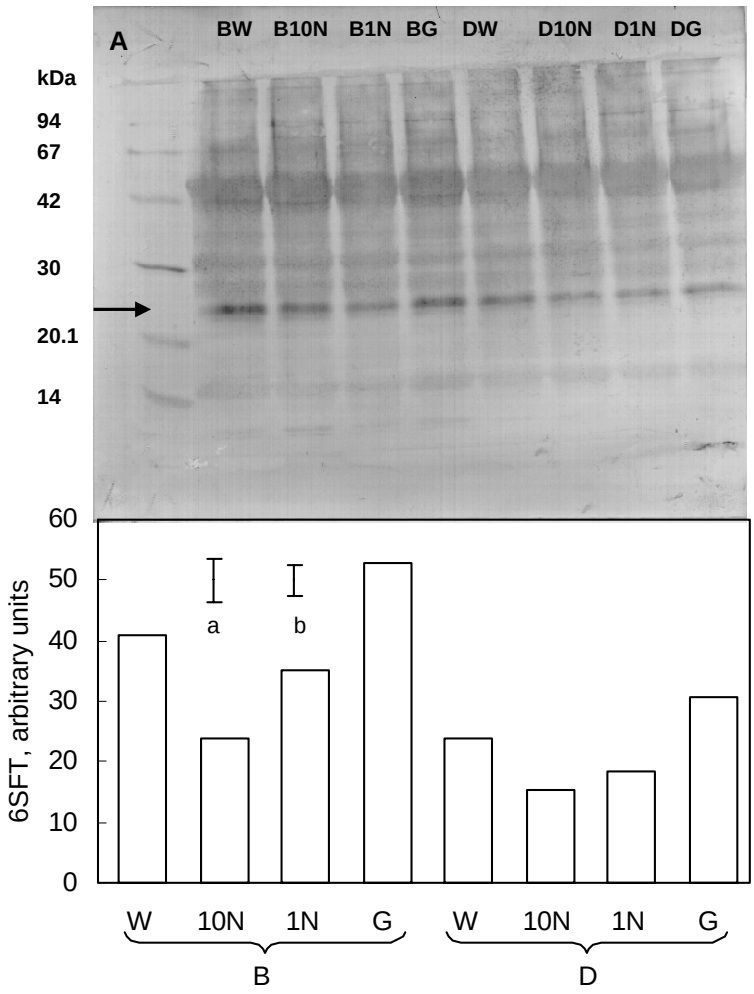
A
BW
B10N
B1N
BG
DW
D10N
D1N
DG
kDa
94
67
42
30
20.1
14
6SFT, arbitrary units
60
50
40
30
20
10
0
a
b
W
10N
1N
G
W
10N
1N
G
B
D

Fig. 5

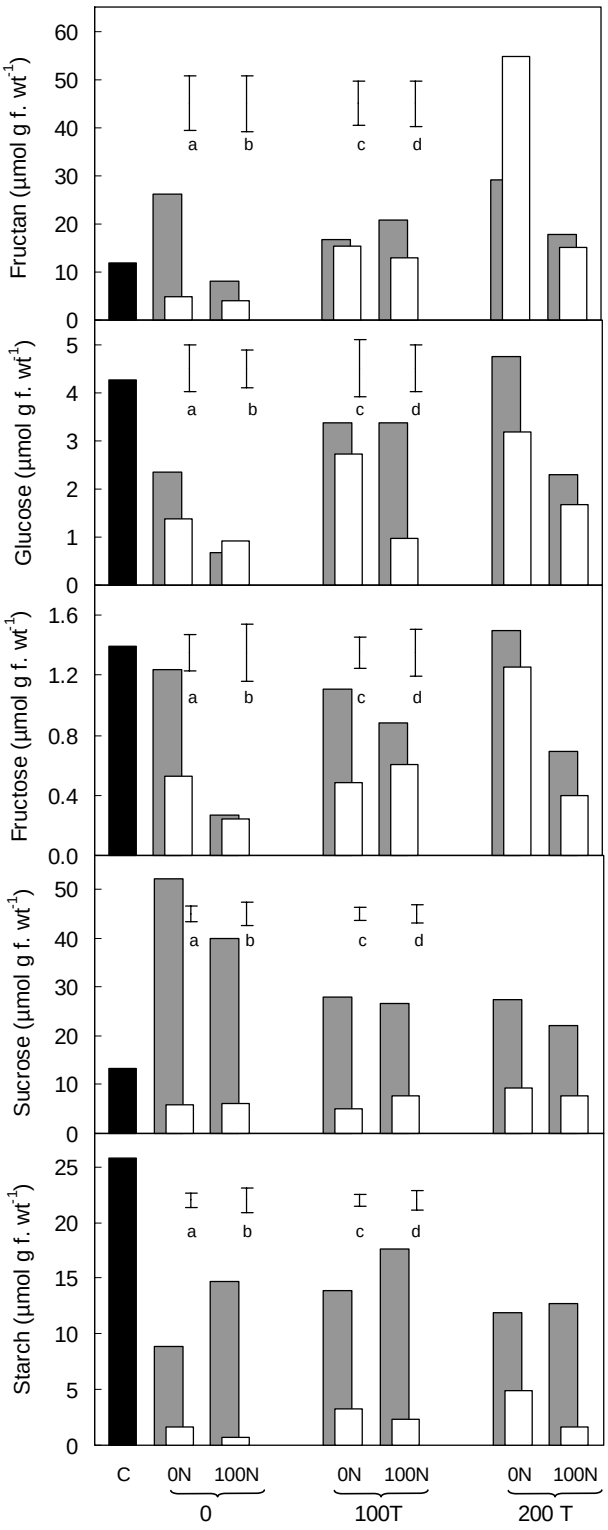
Fructan (µmol g f. wt-1)
Glucose (µmol g f. wt-1)
Fructose (µmol g f. wt-1)
Sucrose (µmol g f. wt-1)
Starch (µmol g f. wt-1)
a
b
c
d
C
0N
100N
0
100T
200 T

Fig. 6

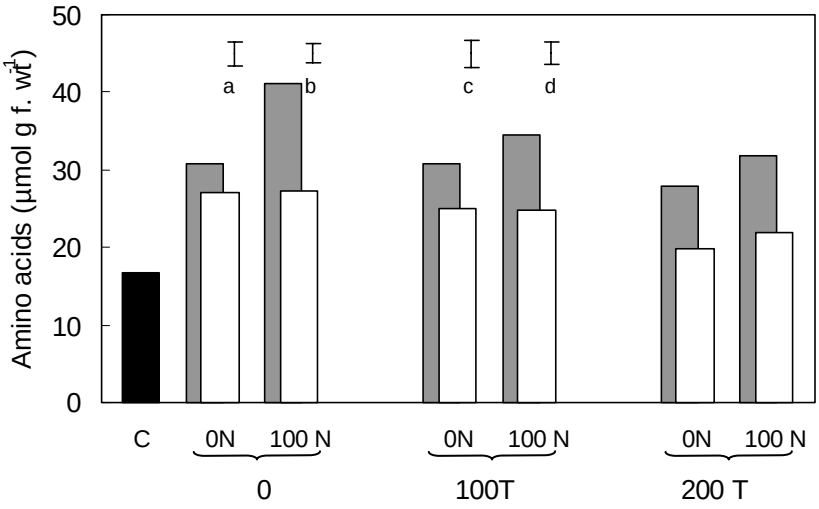
Amino acids (µmol g f. wt-1)
50
40
30
20
10
0
a
b
c
d
C
0N
100 N
0N
100 N
0N
100 N
0
100T
200 T

Fig. 7

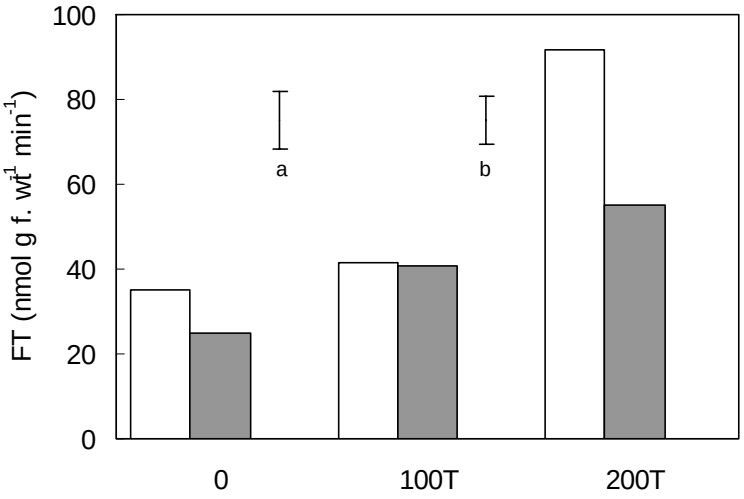

Fig. 8

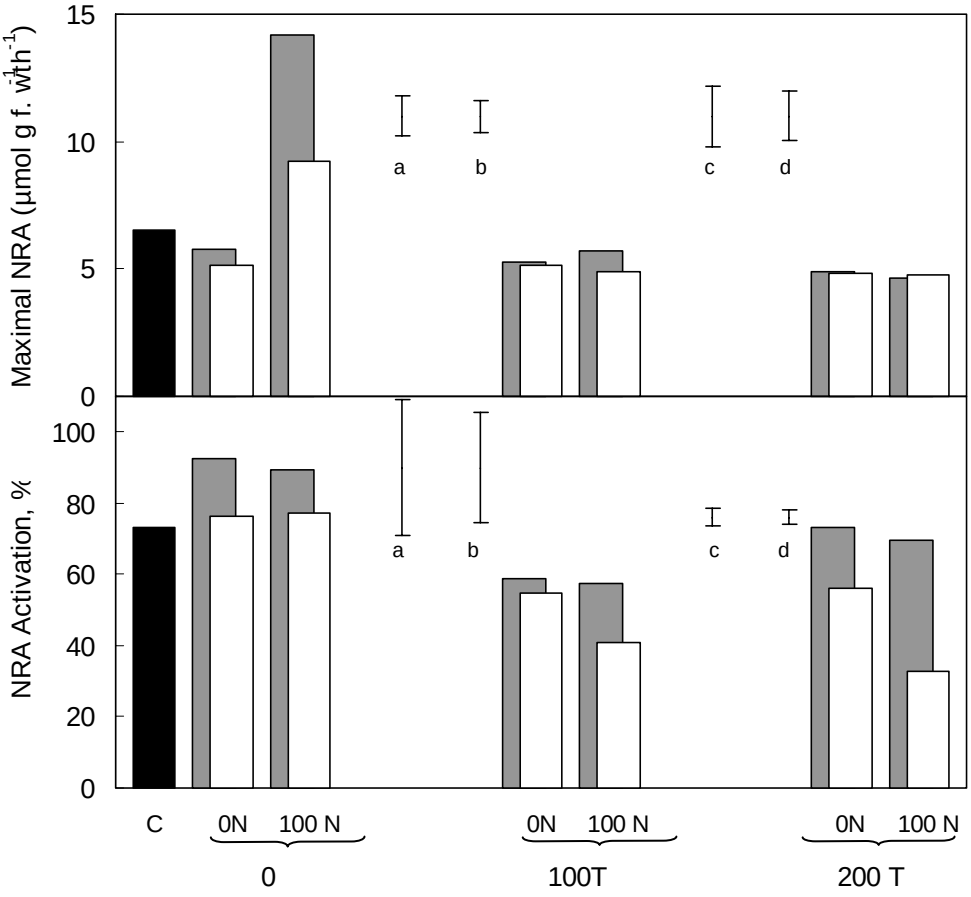
Maximal NRA (µmol g f. wt h-1)
15
10
5
0
NRA Activation, %
100
80
60
40
20
0
a
b
c
d
C
0N
100 N
0N
100 N
0N
100 N
0
100T
200 T

Fig. 9

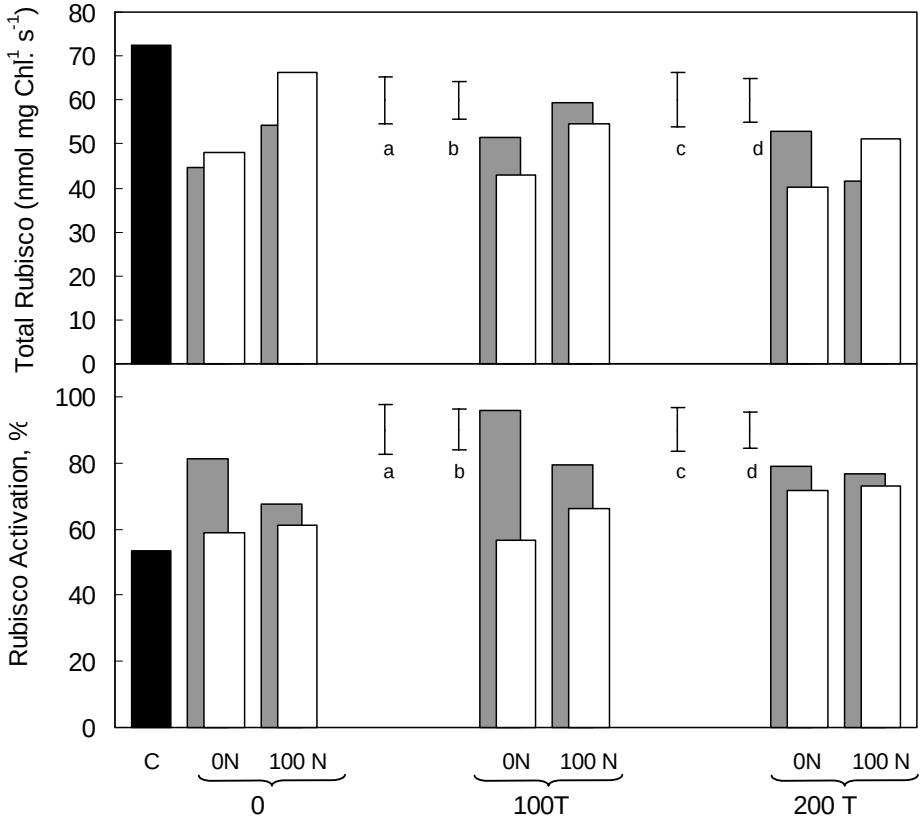
Total Rubisco (nmol mg Chl^{1} s^{-1})
80
70
60
50
40
30
20
10
0
a
b
c
d
Rubisco Activation, %
100
80
60
40
20
0
a
b
c
d
C
0N
100 N
0N
100 N
0N
100 N
0
100T
200 T

Fig. 10

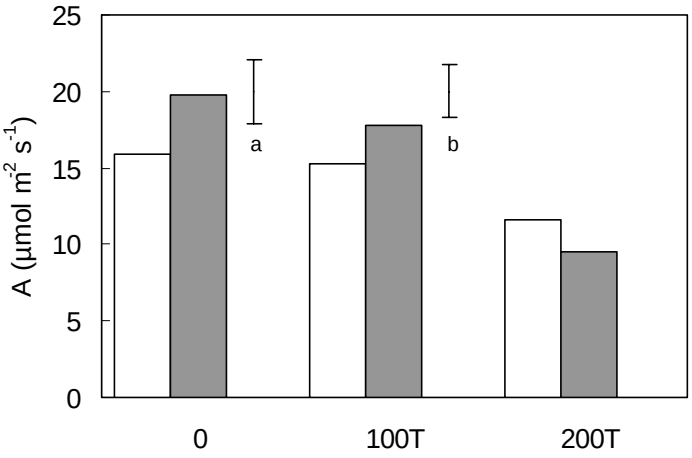

Fig. 11